EXHIBIT A

Cloning and characterization of a *Moraxella bovis* cytotoxin gene

John A. Angelos, MS, DVM; John E Hess, PhD; Lisle W. George, DVM, PhD

Objective—To identify the *Moraxella bovis* cytotoxin gene.

Procedure—Hemolytic and nonhemolytic strains of *M bovis* were compared by use of western blotting to identify proteins unique to hemolytic strains. Oligonucleotide primers, designed on the basis of amino acid sequences of 2 tryptic peptides derived from 1 such protein and conserved regions of the *C* and *B* genes from members of the repeats in the structural toxin (RTX) family of bacterial toxins, were used to amplify cytotoxin-specific genes from *M bovis* genomic DNA. Recombinant proteins were expressed, and antisera against these proteins were produced in rabbits.

Results—Several proteins ranging in molecular mass from 55 to 75 kd were unique to the hemolytic strain. An open reading frame encoding a 927-amino acid protein with a predicted molecular mass of 98.8 kd was amplified from *M bovis* genomic DNA. The deduced amino acid sequence encoded by this open reading frame was homologous to RTX toxins. Antisera against the recombinant carboxy terminus encoded by this open reading frame neutralized hemolytic and cytolytic activities of native *M bovis* cytotoxin.

Conclusions and Clinical Relevance—A gene was identified in *M bovis* that encodes a protein with sequence homology to other RTX toxins. Results of cytotoxin neutralization assays support the hypothesis that *M bovis* cytotoxin is encoded by this gene and belongs in the RTX family of bacterial exoproteins. Identification of this gene and expression of recombinant cytotoxin could facilitate the development of improved vaccines against infectious bovine keratoconjunctivitis. (*Am J Vet Res* 2001;62:1222–1228)

Moraxella bovis is the etiologic agent of infectious bovine keratoconjunctivitis (IBK),[1] the most common ocular disease of cattle. Affected animals develop corneal ulceration and edema, signs of ocular pain, photophobia, and excessive tearing.[2] Pathogenesis of *M bovis* depends on the expression of an exotoxin with calcium-dependent hemolytic, corneotoxic, and leukotoxic properties.[3,4] A single cytotoxin is believed to account for all of these actions[3]; nonhemolytic strains of *M bovis* are not pathogenic for cattle.[5,7] Cattle with IBK develop antihemolysin antibodies[3,8-10] that neutralize the hemolysin from multiple strains of *M bovis*.[10] In addition, calves vaccinated with *M bovis* hemolysin are protected against challenge with a heterologous strain of *M bovis*.[8] Together, these results have established both the importance of *M bovis* cytotoxin in the pathogenesis of IBK and of the host immune response to the cytotoxin.

At the cellular level, exposure of erythrocytes to *M bovis* hemolysin results in potassium efflux, colloid-osmotic cell swelling, and lysis. These effects can be blocked by osmotic protectants, suggesting that the hemolysin forms transmembrane pores in target cell membranes.[11] In a report by Gray et al,[4] the authors hypothesized that *M bovis* cytotoxin may belong to the repeats in the structural toxin (RTX) family of bacterial pore-forming toxins, because a monoclonal antibody against HlyA, an RTX toxin expressed by uropathogenic *Escherichia coli*, recognized a 110-kd protein in extracts from only hemolytic strains of *M bovis*. More recently, a monoclonal antibody was described that neutralizes the hemolytic activity of *M bovis* and recognizes a 94-kd protein from hemolytic *M bovis*.[12]

Exoproteins belonging to the RTX family have been reported in a growing number of gram-negative bacteria, including several important animal pathogens. These include *Mannheimia haemolytica*, the agent of shipping fever pleuropneumonia in cattle, and *Actinobacillus pleuropneumoniae* and *Actinobacillus suis*, the agents of swine pleuropneumonia. Recently, an RTX operon was characterized in *Pasteurella aerogenes*,[13] a cause of abortion in swine and other mammals.

The most well-characterized member of the RTX family is the HlyA protein of *E coli*. Four RTX genes organized in a single operon in the order *C-A-B-D* control production, activation, and secretion of HlyA.[14,15] The structural toxin (product of the *A* gene) is activated by the product of the *C* gene through fatty acylation of conserved lysines.[16-20] The activated toxin is secreted from the bacteria by membrane transport proteins encoded by RTX *B* and *D* genes and a third protein, TolC, that is encoded by a gene lying outside of the RTX operon.[21-23]

Although others have hypothesized that *M bovis* cytotoxin is a member of the RTX family, neither the *M bovis* cytotoxin gene or gene product has been characterized at the molecular level. Thus, the purpose of the study reported here was to identify and clone the *M bovis* cytotoxin gene and determine whether it encodes a protein that belongs to the RTX family of bacterial exoproteins.

Materials and Methods

***Moraxella bovis* strains and culture conditions**—Hemolytic pathogenic strains of *M bovis* (strains T+ and Tifton I) were isolated from 2 beef cows with IBK. A nonhemolytic

Received Aug 25, 2000.
Accepted Dec 12, 2000.
From the Department of Medicine and Epidemiology, School of Veterinary Medicine (Angelos, George), and the Department of Cell Biology and Human Anatomy, School of Medicine (Hess), University of California, Davis, CA 95616.
Supported by University of California School of Veterinary Medicine Formula Fund grants AH-125 and 4979-H.
The authors thank Paul Fitzgerald and Jodi T. Casselman for technical assistance.

mutant strain (T–) arose spontaneously from subcultures of the T+ strain. Isolates were identified as *M bovis* by colony morphology and biochemical criteria.[14] *Moraxella bovis* was propagated on 5% sheep or cow blood agar plates; strains were stored frozen in 50% skim milk/50% glycerol at –80 C.

Purification of *M bovis* cytotoxin—Bacteria from 2 standard 100 X 15-mm blood agar plates heavily streaked with *M bovis* and cultured for 20 hours were harvested, resuspended in trypticase soy broth, inoculated into 500 ml of brain heart infusion (BHI) broth[a] containing 1.5 m*M* $CaCl_2$, and grown for 6 to 8 hours at 35 C on a shaker. Typically, for 1 run of cytotoxin production, 4 L of BHI was inoculated with for each strain of *M bovis* (ie, T+, Tifton, and T–). All purification steps were performed at 4 C unless otherwise noted. Broth cultures were centrifuged for 1 hour at 9,000 rpm,[b] and the supernatant was filtered through a 0.2-µm polyethersulfone membrane.[c] The filtrate was concentrated approximately 20-fold by ultrafiltration with a spiral-wrapped regenerated cellulose cartridge[d] according to recommendations of the manufacturer. The concentrated material was then diafiltered against 45 volumes of chilled (4 C) buffer containing 50 m*M* Tris, 500 m*M* sodium acetate, 1.5 m*M* $CaCl_2$, and 20% glycerol (pH 8.2). When diafiltration was stopped, the optical density (OD; wavelength, 280 nm) of the filter permeate was ≤ 0.01. The material retained by the filter following dialysis was a partially purified cytotoxin-enriched extract that was designated diafiltered retentate; this material was stored at –80 C until purified further by use of column chromatography. Protein concentration in the diafiltered retentate was determined by use of a commercial kit,[e] using bovine serum albumin as the standard.

Results of a previous study[f] indicated that hemolytic and cytolytic activity eluted in the void volume of a gel filtration column.[g] Therefore, frozen aliquots of diafiltered retentate were thawed, concentrated 10- to 15-fold,[h] desalted[i] into fast protein liquid chromatography (FPLC) buffer (50 m*M* Tris, 1.5 m*M* $CaCl_2$, 0.9% NaCl, 5% glycerol [pH 8.0]), filtered through a 0.2-µm filter,[j] and chromatographed on a gel filtration column[k] in FPLC buffer at ambient temperature. This material was referred to as chromatographed diafiltered retentate and was stored at –80 C. Chromatographed diafiltered retentates from both hemolytic and nonhemolytic strains of *M bovis* were subsequently analyzed by use of SDS-PAGE and western blotting to detect proteins unique to the hemolytic strain. In addition, chromatographed diafiltered retentate from the T+ strain was used to immunize rabbits for production of antisera against native cytotoxin.

Production and sequencing of cytotoxin tryptic peptides—Chromatographed diafiltered retentates from the T+ strain of *M bovis* were pooled and concentrated 5- to 10-fold,[h] electrophoresed on a polyacrylamide gel, and stained with Coomassie blue. A 70-kd band unique to the T+ strain was excised from the gel and digested with trypsin. The resulting tryptic peptides were fractionated by use of high-pressure liquid chromatography. The N-terminal amino acids of 2 tryptic peptides, designated peptides 23 and 26, were sequenced at a protein sequencing facility[l] by use of Edman degradation chemistry.

Determination of the cytotoxin gene sequence—All DNA manipulations were performed as recommended by reagant manufacturers or by use of standard methods.[15] Plasmid DNA was isolated and purified by use of a commercial kit.[i] Genomic DNA from *M bovis* strain Tifton 1 was isolated as described[25] and used as the template for polymerase chain reaction (PCR) amplification of the *M bovis* cytotoxin gene. Two degenerate primers, designated 23A (5'-AAY AAA GAR TTR GAR GCN GAR-3') and 26A (5'-CCY TCN CCR CTR TGR AAD ATR TCR TTR AAT TT-3'), were designed by reverse translating the amino acid sequences of peptides 23 and 26, respectively. Additional degenerate primers were designed by reverse translating respective conserved regions of the protein products of the C and B genes of RTX family members from other bacteria; these were designated C-down (5'-ATH GAY TGG ATH GCN CCN TTY GGN GAY-3') and B-up (5'-ACT TTA TCC ATC ACR ACT TGR AAR AA-3'). *Moraxella bovis* genomic DNA was amplified, using each primer pair (23A/26A and C-down/B-up) and *Taq* polymerase.[m] Each amplification consisted of 30 cycles of the following steps: 95 C for 30 seconds, 55 C for 1 minute, and 72 C for 45 seconds (primers 23A/26A) or 2 minutes (primers C-down/B-up). Amplification products were analyzed by use of agarose gel electrophoresis; amplified products were sequenced directly or after cloning into specific sequencing vectors.[n] Additional primers were designed as needed to obtain DNA sequence data for both strands of multiple overlapping regions. Sequence ambiguities were resolved by sequencing PCR products produced by amplification of *M bovis* genomic DNA. Automated DNA sequencing was performed at a DNA sequencing facility,[o] and DNA sequences of overlapping fragments were assembled, using commercially available software,[p] to yield the final complete sequence.

Expression of the recombinant internal peptide of *M bovis* cytotoxin—The PCR product amplified with primer pair 23A/26A was cloned into a general purpose cloning vector,[q] and the resulting recombinant plasmid was digested with *EcoR*I. The appropriate *EcoR*I fragment was gel purified[r] and ligated into an expression plasmid[s] to yield a construct that would direct synthesis of amino acids 438 through 713 of the *M bovis* cytotoxin gene product. This expression plasmid was introduced into competent *E coli* strain DH5α cells,[t] which were then grown to an OD of 0.6 at a wavelength of 600 nm. Expression of the recombinant protein was induced by adding isopropylthio-β-galactoside to 1 mM. Induction was continued for 4 to 6 hours, and cells were harvested by centrifugation. The expressed proteins formed inclusion bodies that were purified as described.[26] Purified inclusion bodies were solubilized in buffer containing 20 mM Tris (pH 8.0), 1 mM EDTA, and 6M urea and chromatographed on a dextran and agarose gel-filtration column.[g] Peak fractions were pooled, dialyzed against PBS solution, and stored at –20 C. This protein, which formed a precipitate following dialysis, was designated the internal peptide.

Expression of the recombinant carboxy peptide of *M bovis* cytotoxin—The complete cytotoxin gene was amplified from genomic DNA of *M bovis* strain Tifton 1, using primers designated *Nde*I-start (5'-GAT CAT ATG TCC AAT ATA AAT GTA ATT AAA TCT AA-3') and B-up2 (5'-ATC ACT AGT TCC ATA ATC TAT AAC CAA TGA-3'). This primer pair allowed for amplification of a fragment from 6 bases upstream of the ATG start codon for the *M bovis* cytotoxin gene to 64 bases downstream of the termination codon. The amplification conditions were as described for cytotoxin gene sequencing except that the elongation step (72 C) was for 1 minute. The resulting PCR product was cloned into a general purpose cloning vector,[q] and the recombinant plasmid was digested with *Xho*I and *EcoR*I. The *Xho*I-*EcoR*I fragment (from the *Xho*I site at position 1,926 of the cytotoxin gene to the *EcoR*I site in the vector) was blunt ended with Klenow polymerase[m] and gel purified. The gel-purified fragment was ligated into an expression plasmid[u] to yield a construct that would direct synthesis of amino acids 643 through 927 of the *M bovis* cytotoxin gene product while adding 4 amino acids (Met-Ala-Arg-Ile) at the N-terminus. The recombinant plasmid was transformed into competent *E coli* strain DH5α cells and purified prior to trans-

formation into *E coli* strain BL21 (DE3)[r] for expression. Transformed bacteria were grown and production of the recombinant peptide was induced as described for the internal peptide. The recombinant peptide formed inclusion bodies that were purified as described.[28] Purified inclusion bodies were solubilized in buffer containing 10 m*M* Tris (pH 8.0), 1 m*M* EDTA, and 8*M* urea and chromatographed on an anion exchange column.[a] Peak fractions were identified by use of SDS-PAGE, pooled, and chromatographed on a gel-filtration column.[a] Peak fractions from the gel-filtration column were pooled, dialyzed against PBS solution, and stored at −20 C. This protein, which remained soluble following dialysis, was designated the carboxy peptide.

Production of polyclonal antibodies against *M bovis* cytotoxin—Antisera for use in western blot and lysis neutralization assays were prepared by immunizing New Zealand white rabbits with chromatographed diafiltered retentate from *M bovis* strain T+ or filter sterilized tryptose broth cultures of *M bovis* strain T+ mixed 1:1 (vol/vol) with Freund's complete adjuvant. Booster vaccinations (antigen mixed 1:1 in Freund's incomplete adjuvant) were performed 21 days later, and serum was obtained 21 days after booster vaccinations. Antiserum against chromatographed diafiltered retentate was designated rabbit anti-column fraction, whereas antiserum against culture filtrates was designated rabbit anti-T+ antiserum. The latter antiserum neutralized hemolytic and cytolytic activity of native *M bovis* cytotoxin and served as a positive control antiserum in lysis neutralization assays.

Polyclonal rabbit antiserum to the recombinant internal (amino acids 438 through 713) and carboxy (amino acids 643 through 927) peptides of *M bovis* cytotoxin was prepared commercially.[v] Before use in western blot analyses, these antisera were preabsorbed with an *E coli* DH5a lysate. Serum was heat inactivated at 56 C for 1 hour prior to use in lysis neutralization assays.

Western blot analyses—Proteins unique to the T+ strain of *M bovis* were detected by use of western blotting. Chromatographed diafiltered retentates from T+ and T− strains were concentrated 5- to 10-fold,[b] mixed with an equal volume of 2X loading buffer (62.5 mM Tris [pH 6.8], 0.7*M* β-mercaptoethanol, 20% glycerol, 4.1% SDS, and 0.2 mg of bromophenol blue/ml), heated to 95 to 100 C for 5 minutes, electrophoresed on a polyacrylamide gel (stacking gel, 3.9% polyacrylamide; running gel, 7.5% polyacrylamide), and transferred to a polyvinylidene fluoride membrane.[w] To block nonspecific binding sites, the membrane was incubated overnight at room temperature on a rotating shaker in blocking buffer (TSN buffer [20 m*M* Tris, 0.9% NaCl, and 0.1% Tween 20] with 5.5% teleostean gelatin; pH 7.4).[x] Membranes were then incubated with rabbit anti-column fraction antiserum diluted 1:400 in fresh blocking buffer overnight at room temperature on a rotating shaker, washed 3 times in TSN buffer, and immersed in a solution containing 0.5 μCi of ^{125}I-labeled Protein A/20 ml of TSN buffer overnight at room temperature on a rotating shaker. After incubation, membranes were washed in TSN buffer and autoradiographed.

To determine whether cytotoxin was produced by both hemolytic and nonhemolytic strains of *M bovis*, strains T+, T−, and Tifton I were inoculated into LB broth containing 1.5 m*M* $CaCl_2$ and incubated while shaking at 250 rpm for 3 to 6 hours at 35 C. Culture supernatants were harvested and analyzed by use of SDS-PAGE and western blotting, using rabbit antisera against the internal or carboxy peptides as the primary antibody.

Lysis neutralization assays—To determine whether antisera against the recombinant carboxy peptide could neutralize hemolytic or cytolytic activity of *M bovis* strain Tifton I, antisera were diluted 1:64 in Tris buffered saline-calcium chloride (TBS $CaCl_2$) buffer (50 m*M* Tris, 150 m*M* NaCl, 1.5 m*M* $CaCl_2$ [pH 7.4]). An equal volume of either TBS $CaCl_2$ buffer or recombinant carboxy peptide (0.1 mg/ml in TBS $CaCl_2$ buffer) was added to diluted antisera. Samples were incubated at room temperature for 30 minutes and then at 37 C for 30 minutes, followed by centrifugation at maximum speed in a microcentrifuge for 10 minutes. Supernatants were harvested, mixed with an equal volume of diafiltered retentate from *M bovis* strain Tifton I diluted 1:32 in TBS $CaCl_2$ buffer, and incubated for 1 hour at 4 C, after which hemolysis or cytolysis neutralization assays were performed.

For hemolysis neutralization assays, 500 μl of a 1% (vol/vol) suspension of washed and pelleted bovine erythrocytes in TBS $CaCl_2$ buffer was added to 500 μl of each test sample (ie, antisera [alone or preabsorbed with carboxy peptide] incubated with diafiltered retentate from *M bovis* strain Tifton I) and incubated for 6 hours at 37 C. Samples were then inverted once, centrifuged for 1 minute at maximum speed in a microcentrifuge, and 350 μl of the supernatant was transferred to a well of a 96-well microtiter plate for spectrophotometric determination of the OD at a wavelength of 455 nm. Negative and positive controls in the 96-well microtiter plate were 350 μl of TBS $CaCl_2$ buffer or diafiltered retentate from strain Tifton I diluted 1:32 in TBS $CaCl_2$ buffer, respectively; a control for the carboxy peptide in TBS $CaCl_2$ buffer was also included. Percent hemolysis was determined, using the formula:

$$\% \text{ hemolysis} = [(OD_{sample} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control})] \times 100$$

Percent neutralization was calculated by subtracting the percent hemolysis from 100. Reported results reflect the mean of values from 3 experiments.

For cytolysis neutralization assays, bovine lymphocyte cells (BL-3 cell line)[s] were cultured in Leibovitz L-15 and minimal essential media[m] (1:1 vol/vol) with penicillin-streptomycin[n] (50 U/ml and 50 μg/ml, respectively) and 15% heat inactivated fetal bovine serum[m] at 37 C in 6% CO_2. Cells were harvested by centrifugation, washed 3 times in PBS solution, and resuspended in McCoys 5A medium[m] to a final concentration of 2×10^7 cells/ml. Cells were labeled with 200 μCi of ^{51}Cr for 1 hour at 37 C while rotating, followed by 3 washes in McCoys 5A medium. After the final wash, labeled cells were diluted in McCoys 5A medium to a final concentration of 4×10^5 cells/ml.

Five hundred microliters of labeled BL-3 cells was added to 500 μl of each test sample (ie, antisera [alone or preabsorbed with carboxy peptide] incubated with diafiltered retentate from *M bovis* strain Tifton I), incubated for 1 hour at 37 C while rotating, and pelleted in a microcentrifuge. Radioactivity (counts per minute [CPM]) was determined in 500 μl of each supernatant. Negative and positive controls were as described for the hemolysis neutralization assay. Percent cytolysis was determined, using the formula:

$$\% \text{ cytolysis} = [(CPM_{sample}) - CPM_{negative\ control})/(CPM_{positive\ control} - CPM_{negative\ control})] \times 100$$

Percent neutralization was calculated by subtracting percent cytolysis from 100. Reported results reflect the mean of values from 3 experiments.

Amino acid sequence analysis—Relationships between the deduced amino acid sequences of full- and partial-length *M bovis* cytotoxin and known sequences were determined, using an online database.[29] Multiple sequence analyses, alignments, and homology calculations were determined, using a computer program.[30]

Results

Amino acid sequences of cytotoxin tryptic peptides—Western blot analysis of chromatographed diafiltered retentate from *M bovis* strains T+ and T−, using rabbit anti-column fraction antiserum, revealed proteins in the 55- to 75-kd range that were unique to the T+ strain (Fig 1). Tryptic peptides were prepared from a prominent 70-kd band in the T+ strain. The amino acid sequences for tryptic peptides 23 and 26 were Phe-Leu-Ser-Glu-Leu-Asn-Lys-Glu-Leu-Glu-Ala-Glu and Phe-Asn-Asp-Ile-Phe-His-Ser-Gly-Glu-Gly-Asp-Asp-Leu-Leu-Asp-Ser-Gly-Ala, respectively. Database searches identified homology between these 2 sequences and the deduced amino acid sequences of the *A* gene of RTX family members from *E coli*, *Mannheimia haemolytica*, *A suis*, *A pleuropneumoniae*, and *Actinobacillus actinomycetemcomitans*. These alignments also predicted that peptide 23 was amino to peptide 26.

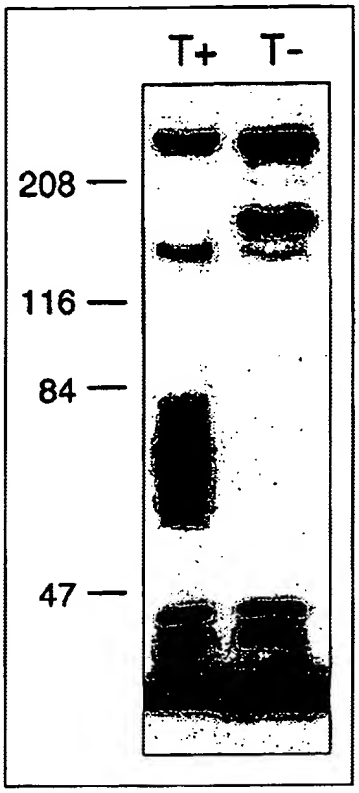

Figure 1—Autoradiogram of partially purified culture supernatants (concentrated chromatographed diafiltered retentates) from hemolytic (T+) and nonhemolytic (T−) strains of *Moraxella bovis*. Concentrated chromatographed diafiltered retentates were electrophoresed on a polyacrylamide gel, and proteins were transferred to a polyvinylidene fluoride membrane that was then incubated with rabbit polyclonal antiserum against chromatographed diafiltered retentate of strain T+ (anti-column fraction antiserum) and ^{125}I-labeled Protein A. Molecular mass markers (kd) are indicated on the left of the figure. The anti-column fraction antiserum recognized a group of proteins (molecular mass, 55 to 75 kd) that were unique to the T+ strain.

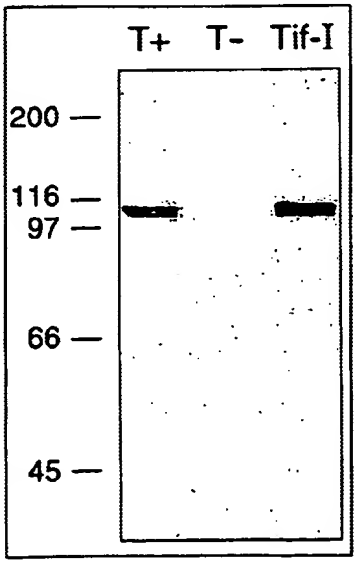

Figure 2—Autoradiogram of 3-hour culture supernatants from *M bovis* strains T+, T−, and Tifton I. Culture supernatants were electrophoresed on a polyacrylamide gel, and proteins were transferred to a polyvinylidene fluoride membrane that was then incubated with rabbit antiserum against the recombinant carboxy peptide of *M bovis* cytotoxin and ^{125}I-labeled Protein A. Molecular mass markers (kd) are indicated on the left of the figure. The anti-carboxy peptide antiserum recognized 2 proteins (molecular mass, 102 and 105 kd) that were unique to the hemolytic strains (T+ and Tifton I).

Cytotoxin gene sequence—Amplification of *M bovis* strain Tifton I genomic DNA with primers 23A and 26A produced an approximately 850-bp fragment. The size of this fragment was consistent with the size predicted on the basis of the locations of the peptide sequences within RTX A proteins. Deoxyribonucleic acid sequencing of the 23A-26A PCR amplicon revealed extensive similarity with other RTX *A* genes.

Amplification of *M bovis* genomic DNA with primers C-down and B-up produced a 4-kb fragment that contained the complete open reading frame of an RTX *A* gene from *M bovis*. The open reading frame was 2,781 nucleotides long and was designated *mbxA*. The nucleotide sequence for *mbxA* has been submitted to the GENBANK database (accession number, AF205359).

Deduced amino acid sequence of *M bovis* cytotoxin—The deduced sequence of the protein encoded by *mbxA* (ie, MbxA or cytotoxin) was 927 amino acids with a predicted molecular mass of 98.8 kd. Percentage of identity of MbxA with the deduced amino acid sequences of other RTX *A* gene products was 50.4% for *Mannheimia haemolytica* LktA,[28] 49.1% for *A pleuropneumoniae* ApxIIA,[29] 48.7% for *A suis* ClyIIA,[31] 43.5% for *E coli* HlyA,[14] and 40.9% for *A actinomycetemcomitans* LtA.[32]

Identification of cytotoxin in *M bovis* culture supernatants—Two proteins with molecular masses of 102 and 105 kd were recognized in 3-hour culture supernatants of strains T+ and Tifton I by use of western blot analysis with rabbit antisera against the carboxy peptide of *M bovis* cytotoxin (Fig 2). These proteins were not detected in culture supernatants of the nonhemolytic T− strain. The 102- and 105-kd proteins were believed to represent native MbxA. However, during longer incubations (4 to 6 hours), smaller immunoreactive proteins with molecular masses ranging from 52 to 91 kd were identified in culture supernatants of *M bovis* strain Tifton I (Fig 3).

Lysis neutralization assays—To determine whether *mbxA* encoded *M bovis* cytotoxin, neutralization assays were performed with rabbit anti-T+ antisera before and after preabsorption with the recombinant carboxy peptide of MbxA. Rabbit anti-T+ antisera lost over 50% of its hemolytic and cytolytic neutralizing capacity following preabsorption with carboxy peptide (Table 1). Two rabbit antisera against the carboxy peptide (sera A and B) neutralized hemolytic and cytolytic activity in the diafiltered retentate of *M bovis* strain Tifton I; the neutralizing

capacity of these sera was equivalent to or greater than the neutralizing capacity of rabbit anti-T+ antisera. In addition, preabsorption of sera A and B with carboxy peptide resulted in a loss of > 60% of the lysis neutralizing capacity of both sera. The use of serum B in cytolysis neutralization assays yielded a percent neutralization value > 100 (107.5%), because cytolysis (CPM) in samples containing serum B was less than in the negative control. The recombinant carboxy peptide of *M bovis* cytotoxin incubated alone with indicator cells was neither hemolytic nor cytolytic.

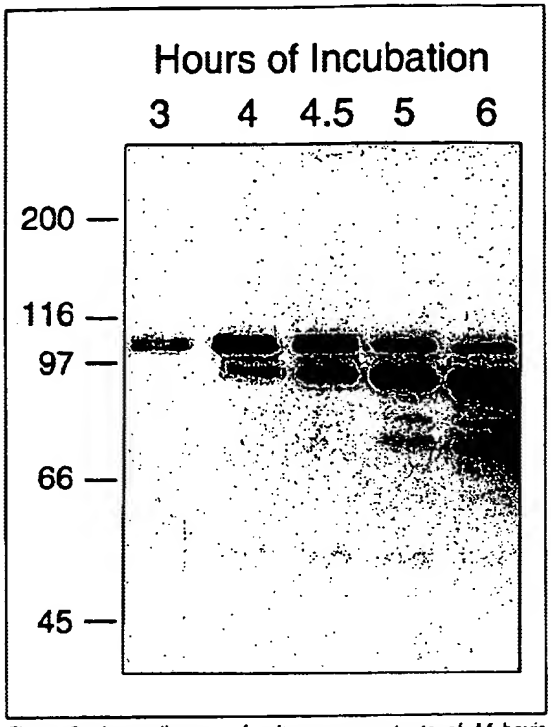

Figure 3—Autoradiogram of culture supernatants of *M bovis* strain Tifton I collected after various incubation times. Culture supernatants were electrophoresed on a polyacrylamide gel, and proteins were transferred to a polyvinylidene fluoride membrane that was then incubated with rabbit antiserum against the recombinant internal peptide (amino acids 438 through 713) of *M bovis* cytotoxin and ^{125}I-labeled Protein A. Molecular mass markers (kd) are indicated on the left of the figure. The anti-internal peptide antiserum recognized 2 proteins (molecular mass, 102 and 105 kd) in the 3-hour supernatants; smaller proteins in supernatants from 4-, 4.5-, 5-, and 6-hour cultures may represent proteolytic products of full-length cytotoxin.

Table 1—Ability of rabbit antisera against the recombinant carboxy terminus of *Moraxella bovis* cytotoxin (MbxA) to neutralize hemolytic and cytolytic activity of native *M bovis* cytotoxin

	Hemolysis		Cytolysis	
Serum	Serum alone	Preabsorbed*	Serum alone	Preabsorbed
Nonimmune†				
Serum A	15.1 ± 2.6	13.2 ± 2.9	18.7 ± 3.6	24.4 ± 8.3
Serum B	2.6 ± 6.7	3.6 ± 2.8	7.5 ± 7.1	10.5 ± 4.0
Immune‡				
Serum A	35.8 ± 2.0	12.8 ± 6.7	76.8 ± 6.8	25.4 ± 6.8
Serum B	66.6 ± 2.6	4.9 ± 4.5	107.5 ± 3.6	15.2 ± 5.6
Anti-T+	36.3 ± 4.8	15.5 ± 1.1	73.9 ± 6.3	23.5 ± 10.1

Data are reported as percent neutralization (mean ± SD), which was determined according to the formula: 100 − {(Result$_{sample}$ − Result$_{negative\ control}$)/(Result$_{positive\ control}$ − Result$_{negative\ control}$)} X 100. *Sera were preabsorbed with the recombinant carboxy peptide (0.1 mg/ml) of *M bovis* cytotoxin. †Nonimmune sera comprised serum from rabbits prior to immunization with the recombinant carboxy peptide of *M bovis* cytotoxin (sera A and B). ‡Immune sera comprised serum from rabbits after immunization with the recombinant carboxy peptide of *M bovis* cytotoxin (sera A and B) or culture filtrates of a hemolytic strain of *M bovis* (anti-T+).

Discussion

This report describes a gene (*mbxA*) in *M bovis* with deduced amino acid sequence homology to RTX toxins. Evidence to support a link between the well-characterized hemolytic activity of cytotoxic strains of *M bovis* and *mbxA* was provided by results of neutralization assays. Antisera against the recombinant carboxy peptide of MbxA neutralized hemolytic and cytolytic activity of native *M bovis* cytotoxin. In addition, the neutralizing capacity of rabbit antiserum to *M Bovis* cytotoxin was reduced following preincubation with a recombinant protein expressed from *mbxA*. Together, these data indicate that *mbxA* encodes a toxin within the RTX family and support the hypothesis that the *M bovis* cytotoxin is encoded by *mbxA*. Moreover, these data are consistent with results of a previous study indicating that a monoclonal antibody against an *E coli* RTX toxin recognized an *M bovis* protein.[4]

To identify *mbxA*, western blotting was performed on purified extracts from hemolytic and nonhemolytic strains of *M bovis* to identify proteins unique to the hemolytic strain. Candidate proteins for *M bovis* cytotoxin were identified, and 1 such protein was selected for amino acid sequencing. The amino acid sequences of 2 peptides derived from this candidate protein had homology to deduced sequences of RTX toxins. Degenerate PCR primers were designed on the basis of the amino acid sequence data, and complete cloning of *mbxA* was subsequently performed, using a PCR-based approach.

The deduced amino acid sequence of the protein encoded by *mbxA* (ie, MbxA) was between 40 and 50% homologous with other RTX toxins; homology extended over the length of the protein. The carboxy terminus of MbxA contains 6 glycine-rich repeats that are characteristic of RTX toxins. Of the 6 repeats, 4 matched the predicted consensus sequence (Leu/Val-Xaa-Gly-Gly-Xaa-Gly-Asn/Asp-Asp-Xaa) for glycine repeats in RTX proteins. It is not known whether *M bovis* cytotoxin requires lysine acylation for activation, as is the case for *E coli* HlyA.[16,17,19,20,33] However, based on alignment of MbxA with HlyA and examination of MbxA for consensus sequences surrounding modified lysines in HlyA,[34,35] we predicted that MbxA requires acylation at lysines 536 and 660 for cytolytic activity.

Results of neutralization assays support the hypothesis that *mbxA* encodes the structural *M bovis* cytotoxin. Cytotoxin-neutralizing antiserum preincubated with the carboxy peptide of *mbxA* lost over 50% of its lysis neutralizing capacity. Furthermore, antisera against the carboxy peptide neutralized hemolytic and cytolytic activities of native *M bovis* cytotoxin. We cannot exclude the possibility that neutralization of hemolysis and cytolysis by anti-carboxy peptide antis-

era was the result of cross-reacting antibodies. Nevertheless, the neutralizing capacity of anti-carboxy peptide antisera was greater than that of antiserum raised against native cytotoxin in *M bovis* culture filtrates (ie, anti-T+ antiserum).

Neutralizing epitopes are also present in the carboxy terminus of other RTX toxins, including HlyA,[36,37] LktA,[38,39] and AC-Hly.[40] The recombinant carboxy peptide expressed from *mbxA* contained 1 of 2 putative lysine acylation sites (lysine 660). This lysine was probably not acylated, because our construct lacked the product of the putative *M bovis* RTX *C* gene; activity of the protein encoded by the *C* gene is considered necessary for activation of RTX toxins.[34,35,41] Thus, our data indicate that acylation of lysine 660 of MbxA is not required for eliciting a neutralizing antibody response against *M bovis* cytotoxin. Such data could be useful when selecting candidate proteins for use in immunizing cattle against IBK.

Proteolysis most likely accounted for the difference in mass between the 70-kd *M bovis* protein selected for amino acid sequencing and the predicted 98.8-kd protein encoded by *mbxA*. Proteolysis was also the likely reason for the appearance of smaller proteins recognized by anti-internal peptide antiserum in 4- to 6-hour culture supernatants of cytotoxic *M bovis*. Hydrolytic enzymes, such as C4 esterase, C8 esterase-lipase, C14 lipase, phosphoamidase, and phosphatase, and proteolytic enzymes, such as leucine and valine aminopeptidases and gelatinase, have been identified in *M bovis* culture supernatants.[42] Our data, however, do not exclude the possibility that *M bovis* also expresses smaller proteins that share epitopes with the internal region of MbxA.

The predicted molecular mass of MbxA is 98.8 kd. However, in the present study, immunoreactive proteins with apparent molecular masses of 102 and 105 kd were detected. We believe that these larger proteins represent full-length MbxA that migrated slower than expected perhaps as a result of acylation of the structural protein. It is also possible that the 105-kd protein is the full-length acylated MbxA protein, and the 102-kd protein is a product of proteolysis. At present, we have no data addressing whether MbxA is acetylated, nor have we extensively characterized the 102/105-kD doublet. We hypothesize that the doublet represents full-length MbxA, and the difference between the predicted and observed mass is attributable to gel artifacts.

In a previous report,[4] a 110-kd protein in concentrated culture supernatants from cytolytic *M bovis* was recognized by a monoclonal antibody against *E coli* HlyA. More recently, a monoclonal antibody that neutralizes hemolytic activity in *M bovis* culture supernatants was shown to recognize a 94-kd protein.[12] We believe that these *M bovis* proteins are likely the same protein encoded by the *mbxA* gene that we describe in the present report. The differences in molecular mass could be explained by proteolysis occurring during harvest and purification of the hemolysin, as well as by variations in experimental protocols.

Moraxella bovis can revert from the hemolytic to nonhemolytic phenotype and vice versa.[7] Loss of the hemolytic phenotype could occur by mechanisms that include mutations that inactivate the toxin, result in loss of an essential accessory function such as activation or secretion of the toxin, or down regulate toxin gene expression, or by loss of plasmids encoding RTX genes. In our experiments, the nonhemolytic T– isolate did not produce proteins that were recognized by antisera against the recombinant internal peptide of MbxA. It is not known whether *mbxA* is absent in the T– strain or whether the gene is mutated or not expressed. Alternatively, the MbxA protein may not be exported. Mutants of *A actinomycetemcomitans* that express variable amounts of leukotoxin have been linked to a 530-bp sequence containing transcriptional control elements.[43] Variability in the hemolytic activity of mutants of *A pleuropneumoniae* were attributed to an inability of nonhemolytic mutants to export toxin.[44] The relationships between any of these mechanisms and loss of hemolytic activity in the T– isolate of *M bovis* is unknown.

Although our data do not define the complete *M bovis* RTX operon, the ability of primers designed from conserved regions of RTX *C* and *B* genes to anneal to *M bovis* genomic DNA suggests that the RTX operon in *M bovis* is organized in a manner consistent with the typical *C-A-B* gene arrangement of RTX operons in other bacterial species. We predict that *M bovis* has a typical RTX operon, and efforts are underway to characterize additional RTX genes in *M bovis*.

---

[a] Difco Laboratories, Detroit, Mich.
[b] Sorvall GS 3 rotor, Kendro Laboratory Products, Newtown, Conn.
[c] Supor 200 filter, Gelman Sciences, Ann Arbor, Mich.
[d] S1Y100 cartridge, Millipore Corp, Bedford, Mass.
[e] BCA protein assay kit, Pierce, Rockford, Ill.
[f] O'Connell KA. *The development and testing of a vaccine for the prevention of infectious bovine keratoconjunctivitis*. PhD Thesis, Department of Medicine and Epidemiology, University of California, Davis, Calif, 1995.
[g] Superose 6 column, Amersham Pharmacia Biotech Inc, Piscataway, NJ.
[h] Centriprep-10, Millipore Corp, Bedford, Mass.
[i] PD-10 column, Amersham Pharmacia Biotech Inc, Piscataway, NJ.
[j] Sterile Acrodisc 13, Gelman Sciences, Ann Arbor, Mich.
[k] Protein Chemistry Laboratory, University of California, Davis, Calif.
[l] Qiaprep Spin miniprep kit, Qiagen, Valencia, Calif.
[m] Life Technologies Inc, Rockville, Md.
[n] pCR2.1-TOPO/TOPO TA cloning kit, Invitrogen Corp, Carlsbad, Calif.
[o] DNA Sequencing Laboratory, University of California, Davis, Calif.
[p] MacDNASIS, Hitachi Software Engineering Co Ltd, Yokohama, Japan.
[q] QIAquick Gel Extraction Kit, Qiagen, Valencia, Calif.
[r] pPro EXHTa, Life Technologies Inc, Rockville, Md.
[s] SuperDex 200 column, Amersham Pharmacia Biotech Inc, Piscataway, NJ.
[t] Novagen, Madison, Wis.
[u] Mono Q column, Amersham Pharmacia Biotech Inc, Piscataway, NJ.
[v] Antibodies Inc, Davis, Calif.
[w] Immobilon P, Millipore Corp, Bedford, Mass.
[x] Sigma, St Louis, Mo.
[y] NEN Life Science Products Inc, Boston, Mass.
[z] Gift of Dr. Gordon Thielen, Department of Surgical and Radiological Sciences, University of California, Davis, Calif.
[aa] Hyclone, Logan, Utah.
[bb] SEQWEB version 1.1 and GCG version 10.1, Genetics Computer Group, Madison, Wis.

References

1. Henson JB, Grumbles LC. Infectious bovine keratoconjunctivitis. I. Etiology. *Am J Vet Res* 1960;761–766.

2. Brown MH, Brightman AH, Fenwick BW, etal Infectious bovine keratoconjunctivitis: a review. *J Vet Intern Med* 1998;12:259–66.

3. Hoien-Dalen PS, Rosenbusch RF, Roth JA. Comparative characterization of the leukocidic and hemolytic activity of *Moraxella bovis*. *Am J Vet Res* 1990;51:191–6.

4. Gray JT, Fedorka-Cray PJ, Rogers DG. Partial characterization of a *Moraxella bovis* cytolysin. *Vet Microbiol* 1995;43:183–96.

5. Beard MK, Moore LJ. Reproduction of bovine keratoconjunctivitis with a purified haemolytic and cytotoxic fraction of *Moraxella bovis*. *Vet Microbiol* 1994;42:15–33.

6. Kagonyera GM, George L, Miller M. Effects of *Moraxella bovis* and culture filtrates on ^{51}Cr-labeled bovine neutrophils. *Am J Vet Res* 1989;50:18–21.

7. Pugh GW Jr, Hughes DE. Experimental bovine infectious keratoconjunctivitis caused by sunlamp irradiation and *Moraxella bovis* infection: correlation of hemolytic ability and pathogenicity. *Am J Vet Res* 1968;29:835–9.

8. Billson FM, Hodgson JL, Egerton JR, et al. A haemolytic cell-free preparation of *Moraxella bovis* confers protection against infectious bovine keratoconjunctivitis. *FEMS Microbiol Lett* 1994; 124:69–74.

9. Nakazawa M, Nemoto H. Hemolytic activity of *Moraxella bovis*. *Nippon Juigaku Zasshi* 1979;41:363–7.

10. Ostle AG, Rosenbusch RE Immunogenicity of *Moraxella bovis* hemolysin. *Am J Vet Res* 1985;46:1011–4.

11. Clinkenbeard KD, Thiessen AE. Mechanism of action of *Moraxella bovis* hemolysin. *Infect Immun* 1991;59:1148–52.

12. Billson FM, Harbour C, Michalski WP, et al. Characterization of hemolysin of *Moraxella bovis* using a hemolysis-neutralizing monoclonal antibody. *Infect Immun* 2000;68:3469–3474.

13. Kuhnert P, Heyberger-Meyer B, Nicolet J, et al. Characterization of PaxA and its operon: a cohemolytic RTX toxin determinant from pathogenic *Pasteurella aerogenes*. *Infect Immun* 2000;68:6–12.

14. Felmlee T, Pellett S, Welch RA. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *J Bacteriol* 1985;163:94–105.

15. Welch RA. Pore-forming cytolysins of gram-negative bacteria. *Mol Microbiol* 1991;5:521–528.

16. Issartel JP, Koronakis V, Hughes C. Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature* 1991;351:759–761.

17. Hughes C, Issartel JP, Hardie K, et al. Activation of *Escherichia coli* prohemolysin to the membrane-targeted toxin by HlyC-directed ACP-dependent fatty acylation. *FEMS Microbiol Immunol* 1992;5:37–43.

18. Hardie KR, Issartel JP, Koronakis E, et al. In vitro activation of *Escherichia coli* prohaemolysin to the mature membrane-targeted toxin requires HlyC and a low molecular-weight cytosolic polypeptide. *Mol Microbiol* 1991;5:1669–1679.

19. Trent MS, Worsham LM, Ernst-Fonberg ML. The biochemistry of hemolysin toxin activation: characterization of HlyC, an internal protein acyltransferase. *Biochemistry* 1998;37:4644–4652.

20. Ludwig A, Garcia F, Bauer S, et al. Analysis of the in vivo activation of hemolysin (HlyA) from *Escherichia coli*. *J Bacteriol* 1996;178:5422–5430.

21. Koronakis V, Stanley P, Koronakis E, et al. The HlyB/HlyD-dependent secretion of toxins by gram-negative bacteria. *FEMS Microbiol Immunol* 1992;5:45–53.

22. Schulein R, Gentschev I, Schlor S, et al. Identification and characterization of two functional domains of the hemolysin translocator protein HlyD. *Mol Gen Genet* 1994;245:203–211.

23. Schlor S, Schmidt A, Maier E, et al. In vivo and in vitro studies on interactions between the components of the hemolysin (HlyA) secretion machinery of *Escherichia coli*. *Mol Gen Genet* 1997;256:306–319.

24. Weyant RS, Wayne Moss C, Weaver RE, et al. *Identification of unusual pathogenic gram-negative aerobic and facultatively anaerobic bacteria*. 2nd ed. Baltimore: The Williams & Wilkins Co, 1996.

25. Sambrook J, Maniatis T, Fritsch EF. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.

26. Nagai K, Thogersen HC. Synthesis and sequence-specific proteolysis of hybrid proteins produced in *Escherichia coli*. *Methods Enzymol* 1987;153:461–481.

27. Tabor S, Richardson CC. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci U S A* 1985;82:1074–1078.

28. BLAST search [online database]. Bethesda, Md: National Center for Biotechnology Information. Available at: http://www.ncbi.nlm.nih.gov. Accessed Sept 9, 1998.

29. Lo RY, Strathdee CA, Shewen PE. Nucleotide sequence of the leukotoxin genes of *Pasteurella haemolytica* A1. *Infect Immun* 1987;55:1987–1996.

30. Chang YF, Young R, Struck DK. Cloning and characterization of a hemolysin gene from *Actinobacillus (Haemophilus) pleuropneumoniae*. *DNA* 1989;8:635–647.

31. Burrows LL, Lo RY. Molecular characterization of an RTX toxin determinant from *Actinobacillus suis*. *Infect Immun* 1992;60:2166–2173.

32. Lally ET, Golub EE, Kieba IR, et al. Analysis of the *Actinobacillus actinomycetemcomitans* leukotoxin gene. Delineation of unique features and comparison to homologous toxins. *J Biol Chem* 1989;264:15451–15456.

33. Stanley P, Koronakis V, Hardie K, et al. Independent interaction of the acyltransferase HlyC with two maturation domains of the *Escherichia coli* toxin HlyA. *Mol Microbiol* 1996;20:813–822.

34. Stanley P, Koronakis V, Hughes C. Acylation of *Escherichia coli* hemolysin: a unique protein lipidation mechanism underlying toxin function. *Microbiol Mol Biol Rev* 1998;62:309–333.

35. Pellett S, Welch RA. *Escherichia coli* hemolysin mutants with altered target cell specificity. *Infect Immun* 1996;64:3081–3087.

36. Ji GE, O'Hanley P. Epitopes of *Escherichia coli* alpha-hemolysin: identification of monoclonal antibodies that prevent hemolysis. *Infect Immun* 1990;58:3029–3035.

37. Oropeza-Wekerle RL, Kern P, Sun D, et al. Characterization of monoclonal antibodies against alpha-hemolysin of *Escherichia coli*. *Infect Immun* 1991;59:1846–1852.

38. Lainson FA, Murray J, Davies RC, et al. Characterization of epitopes involved in the neutralization of *Pasteurella haemolytica* serotype A1 leukotoxin. *Microbiology* 1996;142:2499–2507.

39. Gerbig DG Jr, Cameron MR, Struck DK, et al. Characterization of a neutralizing monoclonal antibody to *Pasteurella haemolytica* leukotoxin. *Infect Immun* 1992;60:1734–1739.

40. Betsou F, Sebo P, Guiso N. The C-terminal domain is essential for protective activity of the *Bordetella pertussis* adenylate cyclase-hemolysin. *Infect Immun* 1995;63:3309–3315.

41. McWhinney DR, Chang YF, Young R, et al. Separable domains define target cell specificities of an RTX hemolysin from *Actinobacillus pleuropneumoniae*. *J Bacteriol* 1992;174:291–297.

42. Frank SK, Gerber JD. Hydrolytic enzymes of *Moraxella bovis*. *J Clin Microbiol* 1981;13:269–271.

43. Brogan JM, Lally ET, Poulsen K, et al. Regulation of *Actinobacillus actinomycetemcomitans* leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. *Infect Immun* 1994;62:501–508.

44. Inzana TJ, Todd J, Ma JN, et al. Characterization of a non-hemolytic mutant of *Actinobacillus pleuropneumoniae* serotype 5: role of the 110 kilodalton hemolysin in virulence and immunoprotection. *Microb Pathog* 1991;10:281–296.